The Product of the DNA Damage-Inducible Gene of *Saccharomyces cerevisiae*, *DIN7*, Specifically Functions in Mitochondria

Marta U. Fikus, Piotr A. Mieczkowski, Piotr Koprowski, Joanna Rytka, Ewa Śledziewska-Gójska and Zygmunt Cieśla

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-106 Warsaw, Poland

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ABSTRACT

We reported previously that the product of the DNA damage-inducible gene of *Saccharomyces cerevisiae*, *DIN7*, belongs to a family of proteins that are involved in DNA repair and replication. The family includes *S. cerevisiae* proteins Rad2p and its human homolog XPGC, Rad27p and its mammalian homolog FEN-1, and Exonuclease I (Exo I). Here, we report that Din7p specifically affects metabolism of mitochondrial DNA (mtDNA). We have found that *dun1* strains, defective in the transcriptional activation of the DNA damage-inducible genes *RNR1*, *RNR2*, and *RNR3*, exhibit an increased frequency in the formation of the mitochondrial petite (ρ^-) mutants. This high frequency of petites arising in the *dun1* strains is significantly reduced by the *din7::URA3* allele. On the other hand, overproduction of Din7p from the *DIN7* gene placed under control of the *GAL1* promoter dramatically increases the frequency of petite formation and the frequency of mitochondrial mutations conferring resistance to erythromycin (E^r). The frequencies of chromosomal mutations conferring resistance to canavanine (Can^r) or adenine prototrophy (Ade^+) are not affected by enhanced synthesis of Din7p. Experiments using Din7p fused to the green fluorescent protein (GFP) and cell fractionation experiments indicate that the protein is located in mitochondria. A possible mechanism that may be responsible for the decreased stability of the mitochondrial genome in *S. cerevisiae* cells with elevated levels of Din7p is discussed.

EUKARYOTIC organisms, such as *Saccharomyces cerevisiae*, disply complex cellular responses to DNA damage, including cell cycle arrest and transcriptional induction of genes involved in DNA replication and DNA damage repair pathways. Several DNA damage-inducible genes (*DIN*) have been identified in *S. cerevisiae*. They include genes encoding S-phase-specific, cell-cycle-regulated proteins involved in DNA synthesis (*POL1*, Johnson *et al.* 1987; *CDC9*, Barker *et al.* 1985; Peterson *et al.* 1985) and nucleotide synthesis (*RNR1*, *RNR2*, *RNR3*, and *CDC8*, Elledge and Davis 1987, 1989; Hurd and Roberts 1989; Elledge *et al.* 1992). In addition to these, several *S. cerevisiae* genes that are known to be involved in DNA repair have been shown to be DNA damage inducible. Known functions of these genes include excision repair (*RAD2*, Madura and Prakash 1986; Robinson *et al.* 1986; *RAD7*, Jones *et al.* 1990; *RAD16*, Bang *et al.* 1995; *RAD23*, Madura and Prakash 1990), recombinational repair (*RAD54*, Cole *et al.* 1987), alkylation repair (*MAG1*, Chen *et al.* 1990), photoreactivation (*PHR1*, Sebastian *et al.* 1990), and repair of DNA cross-links (*SNM1*, Wolter *et al.* 1996). The functions of two sets of genes identified on the basis of increased transcription in response to DNA damage, the *DIN* (Ruby and Szostak 1985) and *DDR* genes (McClanahan and McEntee 1984), remain largely unknown.

We reported previously the cloning and sequence analysis of a novel DNA damage-inducible gene of *S. cerevisiae*, *DIN7* (Mieczkowski *et al.* 1997). By comparison of the predicted Din7p amino acid sequence with those in databases, we found that it belongs to a family of proteins that are involved in DNA repair and replication. The family includes *S. cerevisiae* Rad2p and its *Schizosaccharomyces pombe* and human homologs Rad13p and XPGC (Madura and Prakash 1986; Scherly *et al.* 1993), which are endowed with DNA endonuclease and a weak 5′-3′ exonuclease activity (Habraken *et al.* 1993, 1994) and function in ultraviolet light (UV) excision repair. The strong sequence homologies between Din7p and these proteins are limited to two discrete blocks, designated the N (N-terminal) and I (internal) blocks, interspersed with regions of poor conservation. Another homolog of Din7p is Rad27p of *S. cerevisiae* (Reagan *et al.* 1995; Sommers *et al.* 1995). Mutations in the *RAD27* gene result in a mutator phenotype and increased rates of mitotic crossing over (Reagan *et al.* 1995; Tishkoff *et al.* 1997). The mammalian homolog of Rad27p was identified as 5′-3′ exonuclease required to complete lagging-strand DNA synthesis *in vitro* (Goulian *et al.* 1990; Turchi *et al.* 1994; Waga *et al.* 1994) and as the structure-specific endonuclease FEN-1 proposed to be

Corresponding author: Zygmunt Cieśla, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5A, 02-106 Warsaw, Poland. E-mail: zc@ibbrain.ibb.waw.pl

involved in the processing of branched DNA structures formed by various DNA repair pathways (Harrington and Lieber 1994a,b). The strongest homology to Din7p was found with Exonuclease I (Exo I) of *S. pombe* (Szankasi and Smith 1995) and Exo I (previously designated as Dhs1) of *S. cerevisiae* (Lee *et al.* 1994; Tishkoff *et al.* 1997). Din7p and Exo I of *S. cerevisiae* share a 367-amino-acid domain (56% amino acid sequence identity, 75% similarity). Exo I of *S. pombe*, similar to Din7p (Mieczkowski *et al.* 1997), is induced during meiosis (Szankasi and Smith 1992). *exo1* mutants of *S. pombe* display increased rates of spontaneous mutation and increased meiotic intragenic recombination between close markers, two phenotypes consistent with a role in mismatch correction (Szankasi and Smith 1995). Exo I of *S. cerevisiae* was identified as a protein that interacts with the mismatch recognition protein Msh2 (Tishkoff *et al.* 1997). It was found that *exo1* mutants of *S. cerevisiae* exhibit a mutator phenotype, and epistasis analysis was consistent with Exo I functioning in the Msh2-dependent mismatch repair pathway (Tishkoff *et al.* 1997). Exo I of *S. cerevisiae* was also shown to be required for recombination *in vivo* and *in vitro* (Fiorentini *et al.* 1997). Exo I of both *S. pombe* and *S. cerevisiae* is a 5′-3′ double-stranded DNA-specific exonuclease (Szankasi and Smith 1992; Fiorentini *et al.* 1997).

Although Din7p is remarkably homologous to *S. cerevisiae* Exo I, especially within the highly conserved N and I regions, it is a considerably shorter protein. Moreover, Din7p lacks a domain that is thought to be required for interaction of Exo I with Msh2p (Tishkoff *et al.* 1997). These data suggested that in spite of the very close structural homology between Exo I and Din7p, their physiological roles in *S. cerevisiae* cells might be different.

We show in this study that Din7p specifically affects stability of mitochondrial DNA (mtDNA). We also present evidence that Din7p is located in mitochondria. A possible function of the Din7p in metabolism of mtDNA is discussed.

MATERIALS AND METHODS

Strains: Strains of *S. cerevisiae* used in this study are listed in Table 1. To generate strain MF21 the plasmid pPM19 was used. pPM19 was constructed as follows. First, pYSL1 was digested with *Xho*I and *Cla*I, and the 5.3-kb fragment that contains the *EXO1* gene was ligated into the *Xho*I-*Cla*I site of pGEM7 (Promega, Madison, WI). The resulting plasmid, pPM18, was digested with *Sal*I and *Eco*RI, which removes the internal 1.2-kb fragment of *EXO1*, and the protruding DNA termini was converted to blunt ends by treatment with mung bean nuclease. Subsequently, plasmid pFL34 was digested with *Bgl*II, and the resulting 3′ recessed ends of the 1.1-kb fragment containing *URA3* were filled with the Klenow fragment of DNA polymerase I. The fragment was used to replace the deleted fragment of *EXO1* of the plasmid pPM18, generating plasmid pPM19. Subsequently, pPM19 was digested with *Xho*I and *Cla*I, and the resulting 4.9-kb fragment containing the *exo1::URA3* disruption was transformed into SSL204. The correct integration of the *exo1::URA3* allele in Ura$^+$ transformants was verified by PCR analysis of genomic DNA preparations with primers complementary to sequences within the *URA3* gene and upstream to the disruption of the *EXO1* locus. The partial deletions of the *DIN7* open reading frame in strains Y286 and Y300, generating strains MF23 and MF24, respectively, were constructed by transplacement with a DNA fragment carrying the *URA3* gene, according to the protocol described previously (Mieczkowski *et al.* 1997). The correct integration of the *din7::URA3* allele was verified by PCR analysis of genomic DNA preparations with primers complementary to sequences upstream and downstream of the disruption of the *DIN7* locus. The resulting PCR fragments were tested for the presence of *URA3* by digestion with *Eco*RV.

Growth media and growth conditions: YP medium contained 1% yeast extract and 1% Bacto peptone and 2% dextrose (YPD), 2% glycerol (YPG), or 2% galactose (YPGal). YNB medium contained 0.67% yeast nitrogen base without amino acids and 2% dextrose (YNBD), 2% glycerol (YNBG), or 2% galactose (YNBGal). All YNB media were supplemented with the appropriate nutritional requirements. Solid media contained 2% Bacto agar. Cells were grown at 30°. Petite colonies were scored on YPG medium supplemented with 0.1% glucose (YPGP). For adenine-requiring strains, it was also possible to score petites by the white colony color *vs.* red colony color that *ade1* and *ade2* strains develop. Erythromycin-resistant mutants (E^r) were scored on YPG medium containing 4 mg/ml erythromycin; canavanine-resistant mutants (Can^r) were selected on YNB medium supplemented with 60 μg/ml canavanine sulfate.

Plasmids: The plasmid pPK3, which carries the *DIN7* gene under control of the *GAL1* promoter, was constructed in three steps as follows. First, the vector plasmid pPM29 was made by reconstruction of the plasmid pYES2 (Invitrogen, Carlsbad, CA). pYES2 was digested with *Cla*I, and the resulting 3′ recessed ends were filled with the Klenow fragment. Second, the 1.5-kb *Sma*I-*Eco*ICRI fragment containing the *kanMX4* cassette derived from the plasmid pFA6a-kanMX4 (Eurofan BO Program) was cloned into pYES2 prepared as described above, generating pPM29. Third, the plasmid pPK2 was constructed with the use of a PCR-amplified DNA fragment containing the entire *DIN7* coding sequence. This fragment was generated by PCR with the primers 5′-AACGAAGT**CATATG**GGAATACCTGGCTTAC-3′ and 5′-TTATATG**GCTAGC**GAAAATTGATGGTACGGTGC-3′ and genomic DNA as the template. Underlined bases represent *DIN7* sequences and boldface bases represent the *Nde*I or *Nhe*I restriction sites, respectively. The PCR product was digested with *Nde*I and *Nhe*I and ligated into the *Nde*I-*Nhe*I site of pET27b(+) (Novagen), generating pPK2. Finally, a PCR fragment containing the *DIN7* coding sequence was prepared with the primers 5′-ATAATA**GGATCC**ATGGGAATACCTGGCTTACTG-3′, in which underlined bases represent the *DIN7* sequence, and 5′-ATAATA**GGATCC**TCAGTGGTGGTGGTGGTG-3′, in which underlined bases represent the pET27b multicloning site sequence. Boldface bases represent the *Bam*HI restriction site. The pPK2 DNA preparation was used as the template. The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of pPM29. That pPK3 contains the wild-type *DIN7* sequence fused at the C terminus to the HSV-tag and His-tag sequences was determined by DNA nucleotide sequencing.

The plasmid pPM39 encoding the Din7-GFP fusion protein was constructed as follows. First, a PCR-amplified DNA fragment containing the entire *GFP* sequence and the *kanMX6* cassette flanked by the C-terminal part of *DIN7* and by the sequence downstream of the *DIN7* locus was generated. The plasmid pFA6a-GFPMT-kanMX6 DNA (Eurofan BO Program)

TABLE 1

***S. cerevisiae* strains used in this study**

Strain	Genotype	Reference
FF18733	*MAT***a** *leu2 trp1 ura3 his7-2 lys1-1*	F. Fabre
FFD1	*MAT***a** *dun1::LEU2 leu2 trp1 ura3 his7-2 lys1-1*	F. Fabre
FY1679	*MAT***a**/*MATα ura3-52*/*ura3-52 leu2Δ1*/+*trp1Δ63*/+*his3Δ200*/+	Eurofan BO
SSL204	*MAT***a** *his3-Δ200 leu2 trp1 ura3-52 ade2-101*	R. Malone
Y286	*MATα can1-100 ade2-1 his3-11 leu2-3 trp1-1 ura3-1dun1-Δ100::HIS3*	Zhou and Elledge (1993)
Y300	*MATαcan1-100 ade2-1 his3-11 leu2-3 trp1-1 ura3-1*	Navas *et al.* (1995)
MF21	As SSL204 *exo1::URA3*	This study
NF23	As Y300 *din7::URA3*	This study
MF24	As Y286 *din7::URA3*	This study

was used as the template for PCR with the primers 5′-GACCTCACCAGCAGCGATCTCAGGCACCGTACCATCAATTTTCTCGTACGCTGCAGGTCGAC-3′, in which underlined bases represent the C-terminal *DIN7* sequence and the remainder is the N-terminal *GFP* sequence, and 5′-ATGAAAATATTACAACAATACCTTCGAACATATATATACACCACATCGATGAATTCGAGCTC-3′, in which underlined bases represent the sequence downstream of the *DIN7* locus and the remainder is the C-terminal part of the *kanMX6* cassette sequence. This PCR fragment was introduced into FF18733 by transformation, and clones resistant to G-418 were selected. The correct integration of the fusion was verified by PCR analysis of genomic DNA with primers complementary to sequences upstream and downstream of the *DIN7* locus. Since no fluorescence was found with the strain FF18733 carrying the *DIN7::GFP* fusion, it was decided to place the fusion under control of the *GAL1* promoter. A PCR-amplified fragment containing the entire *DIN7* coding sequence fused to the *GFP* sequence was generated with the primer 5′-ATGGGAATACCTGGCTTACTGCC-3′ representing the N-terminal *DIN7* sequence and the primer 5′-ATGAAAATATTACAACAATACCTTCGAACATATATATACACCACATCGATGAATTCGAGCTC-3′ in which underlined bases represent the sequence downstream of the *DIN7* locus. The genomic DNA preparation from the strain FF18733 *DIN7::GFP* was used as the template. The 3.5-kb PCR fragment was cloned into the *Eco*ICRI restriction site of pYES2, generating the plasmid pPM39. The plasmid pFL34 was provided by F. Lacroute, the plasmid pYSL1 by M. Yamazaki, and the plasmids pZZ48(*DUN1*), pZZ99(*dun1-20*), pRS426, pBAD71(*RNR1*), and pSE324(*RNR2*) by S. Elledge.

Cell fractionation: Cell fractionation was performed by the method described by Daum *et al.* (1982). A total of 100 ml of Y300 strain harboring either the plasmid pPK3 or vector plasmid pPM29 was grown in YPG medium supplemented with G-418 (0.1 mg/ml) to mid-log phase. Then the cells were harvested, suspended in 250 ml YPGal medium, and grown for 6–8 hr to OD_{600} = 1.0. Where indicated, mitochondria were treated for 30 min at 4° with proteinase K (0.4 mg/ml) to remove cofractionating proteins. Proteinase K was inactivated by addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 2 mm.

Immunoblot analysis: Proteins were resolved on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels and transferred to PVDF membrane (msi). The presence of Din7p fused at the C terminus to the HSV-tag was identified with the use of the HSV-tag antibody (Novagen) as a primary antibody and anti-mouse IgG AP conjugate (Promega) as a secondary antibody. The presence of ferrochelatase was identified with the use of antiferrochelatase antibody, which was a gift of J. M. Camadro, and the presence of hexokinase was detected with the use of antihexokinase antibody, which was a gift of G. Schatz. Anti-rabbit IgG AP conjugate (Promega) was used in this case as a secondary antibody. Antibodies were detected with the use of X-Phosphate/NBT (Boehringer Mannheim, Mannheim, Germany).

Partial purification of Din7p: *S. cerevisiae* MF21 carrying either pPM29 or pPK3, which carries *DIN7* sequence fused at the C terminus to the HSV- and hexahistidine-tag sequences, was grown at 30° in YNBG medium supplemented with G-418 (0.1 mg/ml) to OD_{600} = 0.8. Subsequently, galactose was added to the medium, and the cells were grown for an additional 6 hr. The cells were collected by centrifugation, washed once with water, and then resuspended in a volume of cell breakage buffer, pH 7.8 (50 mm Na phosphate, 300 mm NaCl, 10% glycerol, 2 mm imidazole, 1 mm dithiothreitol [DTT], 1 mm PMSF). The resuspended cells were quickly frozen and stored at −80° until use. All of the purification steps were performed at 4°, and 1 mm PMSF was added to all the buffers. Cells (8 g wet weight) were thawed in a mixture of ice and water and disrupted with glass beads. The cell debris was removed by centrifugation at 15,000 rpm in Sorvall SS-34 rotor for 45 min. A total of 0.5 ml of Ni-NTA agarose (Qiagen, Hilden, Germany) was added to 10 ml of the resulting supernatant, and after 45 min the resin was used to form a column. Proteins bound to Ni-NTA were eluted with a step gradient of imidazole. The collected fractions were tested for the presence of Din7p. Under the experimental conditions used, a vast majority of Din7p was eluted with 50 mm imidazole. For nuclease assays the fractions were concentrated and dialyzed by ultrafiltration with the use of Microcon 30 filter (Amicon, Beverly, MA).

Nuclease assays: Uniformly labeled ^{3}H-λ DNA digested with *Hinc*II was used to detect nuclease activity. To prepare ^{3}H-λ DNA, *Escherichia coli* MG1655 strain lysogenic for λ*I857S7* was grown at 30° in minimal medium supplemented with 1% casamino acids. [^{3}H]Thymidine (10 μC/ml) was added at the mid-logarithmic phase, and the culture was shifted to 37° for 4 hr. The labeled DNA was purified with the use of Qiagen Lambda kit. Reaction mixture (40 μl) contained 1 nmol (5100 cpm) of substrate, 6 mm Tris-HCl, pH 7.8, 6 mm $MgCl_2$, 1 mm DTT, 6 mm NaCl, and 5 μl of Ni-NTA column fraction. After incubation at 30° for 1 hr, the reactions were terminated by the addition of 10 μg of salmon sperm DNA and 50 μl 10% trichloroacetic acid (TCA). The mixtures were held on ice for 15 min, and after centrifugation aliquots of supernatants (80 μl) were removed and radioactivity was measured by scintillation counting.

Determination of mutation frequencies: To measure the frequencies of petite formation, single yeast colonies from

YPG plates were inoculated into 4 ml YPG liquid medium and grown to 5–8 × 10^7 cells/ml. Depending on the experiment, 10^2 or 10^3 of these cells were inoculated into 4 ml YPD medium and grown at 30° to the stationary phase. Appropriate dilutions were plated on YPGP, and the percentage of petites was scored after 3 days at 30°. Ten independent cultures of each strain were used. The median value from each set of 10 cultures was used to determine the percentage of petites. To measure mutation frequencies in strains carrying pPK3 or pPM29 plasmids, single yeast colonies on YPD plates were inoculated into 4 ml YNBGal medium and grown to the stationary phase at 30°. Ten independent cultures of each strain were used. Appropriate dilutions were plated on YPD and YNBD − adenine or YNBD + canavanine or YNBD + erythromycin. The median value from each set of 10 cultures was used to determine the frequency of reversion to Ade^+ or the frequency of forward mutation to canavanine or erythromycin resistance.

Staining and microscopy: DNA was stained by the addition to the logarithmically growing cells of 1 μg/ml DAPI. After 1 hr with shaking, cells were centrifuged and resuspended in PBS-glycerol. Microscopy was performed using a Nikon Microphot-SA microscope. For DAPI-stained cells, a UV-2A Nikon filter set was used. For Din7-GFP fluorescence, a high QFITC LP filter was used.

RESULTS

The frequency of petite formation is increased in the *dun1* mutants: The mutants disrupted for *DIN7* are as sensitive to DNA-damaging agents as the wild-type strains, and the rates of spontaneous and induced mutations are the same as in the *DIN7* parental strains (Mieczkowski *et al.* 1997). Interestingly, however, a high basal level of *DIN7* expression was found in strains in which the *DUN1* gene was inactivated. Dun1 protein kinase (Zhou and Elledge 1993) is required for induction of the *RNR1*, *RNR2*, and *RNR3* genes encoding three different subunits of ribonucleotide reductase (Elledge and Davis 1987, 1989; Elledge *et al.* 1992). We hypothesized that a cellular function of Din7p may be somehow related to the regulatory function of Dun1p. Therefore, various growth properties of the *dun1* mutants were examined. One observation seemed to be promising. When strain Y300 *DUN1* was grown on YPD plates, a vast majority of arising colonies were red, and only a few white and small colonies were formed. In contrast to Y300 *DUN1*, white and small colonies appeared frequently when strain Y286 *dun1-Δ100::HIS3* grew under the same conditions. It is well known that certain adenine-requiring *S. cerevisiae* strains produce a red pigment and form red colonies, provided that the cells can respire. Cells that have lost the ability to respire form white colonies (Reaume and Tatum 1949). White colonies formed by Y286 *dun1-Δ100::HIS3* were indeed unable to grow on media containing a nonfermentable carbon source such as glycerol or ethanol. This observation suggested that inactivation of *DUN1* results in the increased production of mitochondrial petite mutants (Dujon 1981).

To compare quantitatively the frequency of petite mutants arising in Y286 *dun1-Δ100::HIS3* and Y300 *DUN1*, the strains were grown for ~15 generations in YPD medium. Then, the cells were plated on YPGP medium, and the frequencies of petites were monitored after 3 days. Figure 1 shows that, under experimental conditions used, formation of petites in Y286 is approximately threefold more frequent than in Y300. Figure 1 also shows that introduction of the plasmid pZZ48, carrying the wild-type *DUN1* gene, but not of the plasmid pZZ99, carrying the *dun1-20* allele encoding an inactive protein kinase, decreases the frequency of petites in Y286 *dun1-Δ100::HIS3* to the level found with Y300 *DUN1*. Similar results to those shown in Figure 1 were obtained when the frequencies of petite formation were determined in the *dun1* and *DUN1* strains of a different genetic background, *e.g.*, FFD1 *dun1::LEU2* and FF18733 *DUN1* (data not shown).

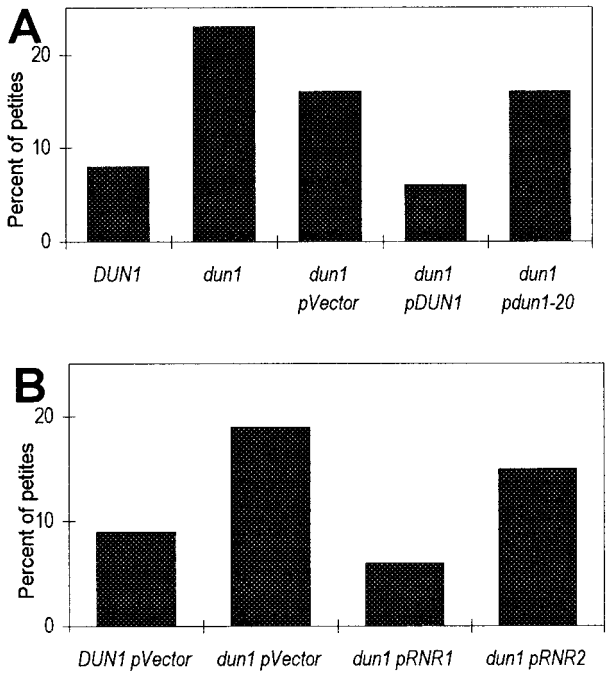

Figure 1.—*dun1* increases the frequency of petites. (A) Strains Y300 *DUN1*, Y286 *dun1-Δ100::HIS3*, and Y286 harboring either the vector plasmid pRS314, pZZ48(*DUN1*), or pZZ99(*dun1-20*) were grown for ~15 generations in YPD medium, and the percentage of petites was estimated as described in materials and methods. Twelve independent cultures of each strain were used. The median value from each set of 12 cultures was used to calculate the percentage of petites. (B) Strains Y300 *DUN1* carrying the vector plasmid pRS426 and Y286 harboring either pRS426, pBAD71 (*RNR1*), or pSE324 (*RNR2*) were grown as described in A. The median value was calculated from 10 independent cultures of each strain.

Petite mutants often result from extensive deletions of the wild-type mtDNA sequence (for review see Dujon 1981). Classical tests were performed to better characterize petites produced by the Y286 *dun1-Δ100::HIS3* strain. About 50 petites were tested by crosses to mit^- tester strains carrying *oxi1*, *oxi2*, or *oxi3* mitochondrial mutations. The results of the crosses revealed a differen-

tial retention by the petite clones of the mitochondrial markers, suggestive of random deletions in, rather than total loss of, mtDNA. Random isolates of the petites were examined also by fluorescence microscopy with the DNA-specific dye, 4′,6′-diamidino-2-phenylindole (DAPI). It appeared that in cells derived from the petite colonies small spots of mtDNA nucleoids surrounding the brightly staining nuclear DNA were seen. Taken together, these results suggest that the majority of petites produced by the *dun1-Δ100::HIS3* mutant contain defective mtDNA molecules and are, therefore, ρ^- but not ρ^0 petites.

Since the basal levels of *RNR1* and *RNR2* expression seem to be decreased in the *dun1* mutants (Zhou and Elledge 1993), it was reasonable to assume that a lower cellular concentration of dNTPs might be responsible for the increased frequency of petites produced by the *dun1* strains. To examine this possibility, the plasmid pBAD71 carrying the *RNR1* gene, the plasmid pSE324 carrying the *RNR2* gene, or the vector plasmid pRS426 was introduced into the Y286 *dun1-Δ100::HIS3* strain, and the frequences of petite formation were determined. Figure 1 shows that the additional copies of the *RNR1* gene decreased the frequency of petite formation in Y286 to the level observed with the wild-type strains Y300. Extra copies of the *RNR2* gene had only a slight effect on petite formation in Y286. The suppression of mtDNA instability by increased dosage and probably increased expression of the *RNR1* gene suggests that the decreased dNTP pools are indeed responsible for this phenotype of the *dun1* mutants.

The elevated level of petite formation in *dun1* strains is partially suppressed by *din7*: The lower levels of dNTPs in *dun1* strains might directly result in defective replication of mtDNA, and in consequence, in the decreased stability of mtDNA. Alternatively, the decreased concentration of dNTPs might affect the frequency of recombination of mtDNA, leading to the more frequent formation of petite genomes. On the other hand, it was reasonable to assume that it is the lower level of dNTPs that triggers the derepression of *DIN7* in the *dun1* mutants (Mieczkowski *et al.* 1997). This assumption was confirmed by our finding that the derepression of *DIN7* is significantly reduced in the *dun1* mutants by the pBAD71 (*RNR1*) plasmid (M. Fikus, unpublished results). We considered the possibility that at least a fraction of petites could arise in *dun1* by the Din7p-mediated process. To examine this possibility, strains disrupted for the *DIN7* gene in either the *DUN1* or *dun1* background were constructed. These strains, MF23 *DUN1 din7::URA3* and MF24 *dun1-Δ100::HIS3 din7::URA3*, and their parental strains, Y300 *DUN1* and Y286 *dun1-Δ100::HIS3*, were grown for ~20 generations in YPD medium and were monitored for the frequencies of petite formation. Figure 2 shows that in the *dun1* background disruption of the *DIN7* gene decreases the production of petites by ~30%. This difference was highly

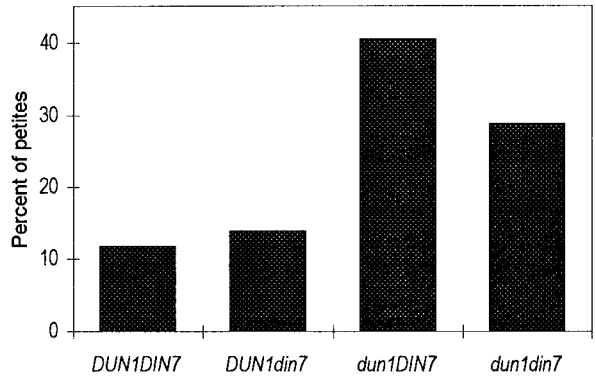

Figure 2.—*din7* decreases the frequency of petites in *dun1* strains. Strains Y300 *DUN1*, MF23 *DUN1 din7::URA3*, Y286 *dun1-Δ100::HIS3*, and MF24 *dun1-Δ100::HIS3 din7::URA3* were grown for ~20 generations in YPD medium, and the percentage of petites was determined as described in materials and methods. For each strain the median value was calculated from 10 independent cultures.

reproducible in four independent experiments. On the other hand, Figure 2 shows also that the *din7::URA3* mutation does not affect the frequency of petites in Y300 *DUN1*. These results suggest that a fraction of petite (ρ^-) mutants formed by the *dun1* strains is generated by the Din7p-mediated process.

Overproduction of Din7p increases the frequency of petites and of E^r mutants: Since inactivation of *DIN7* significantly reduces the frequency of petite mutants produced by the *dun1* strain, one can expect that overexpression of *DIN7* should result in the opposite phenotype. To verify this prediction, strain SSL204 carrying either plasmid pPK3 (*GAL1-DIN7*) or vector plasmid pPM29 was grown for ~15 generations in YNBGal medium. The frequencies of petites and of the mutants resistant to erythromycin (E^r) were determined. Figure 3 shows that overexpression of *DIN7* dramatically enhances the production of petites. When samples of these petites were tested by crosses to *mit*$^-$ tester strains carrying *oxi1*, *oxi2*, or *oxi3* mutations, a differential retention by the petite clones of the mitochondrial markers was found, suggestive of random deletions of mtDNA. In the vast majority of cells derived from these petites, DAPI staining revealed, in addition to strong nuclear fluorescence, weaker peripheral spots representing the multiple molecules of mtDNA (data not shown). Therefore, we conclude that petites formed by the SSL204/pPK3 strain overproducing Din7p, similar to petites formed in the *dun1* strains, are predominantly ρ^- and not ρ^0. Figure 3 shows also that in cells carrying pPK3 the frequency of the mitochondrial E^r mutations increases by ~30-fold compared with cells carrying the vector plasmid pPM29. In contrast, the frequencies of chromosomal mutations conferring either resistance to canavanine (Can^r) or adenine prototrophy (Ade^+) are not increased in cells overproducing Din7p. Taken to-

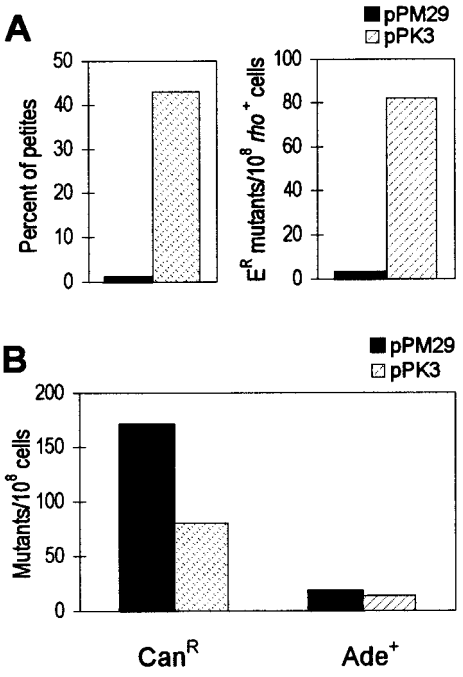

Figure 3.—Overproduction of Din7p increases the frequency of mitochondrial mutations. (A) Strain SSL204 harboring either the vector plasmid pPM29 or the plasmid pPK3 (*GAL1-DIN7*) was grown for ~15 generations in YNBGal medium supplemented with the appropriate nutritional requirements. The percentage of petites and the frequencies of E^r mutants were calculated as described in materials and methods. (B) The frequencies of chromosomal mutations, Can^r and Ade^+, were determined in the same cultures of SSL204/pPM29 and SSL204/pPK3 as described in materials and methods. For each phenotype scored, the median value was calculated from 10 independent cultures of each strain.

gether, these results indicate that an elevated level of Din7p specifically decreases stability of mtDNA.

Din7p is located in mitochondria: The results showing that the cellular level of Din7p specifically affects metabolism of mtDNA strongly suggested that the protein in located in mitochondria. To verify this prediction, plasmid pPM39 encoding Din7p fused at its C terminus to the green fluorescent protein (GFP) of *Aequorea victoria* (Prescott *et al.* 1997) was constructed. Similarly as with pPK3, the hybrid gene *DIN7-GFP* was placed in pPM39 under control of the *GAL1* promoter. To test whether the Din7-GFP fusion protein retains the biological function of Din7p, strains FY1679 harboring either pPM39 or vector plasmid pYES2 was grown in YNBGal medium, and the cultures were monitored for the formation of petites. It appeared that FY1679 carrying pPM39 produced petites at an increased frequency compared with the strain harboring pYES2, suggesting that the fusion Din7-GFP protein retains biological activity of Din7p. To examine subcellular localization of Din7p, strain FY1679 harboring pPM39 was grown to mid-logarithmic phase in YPG medium, and galactose was added to the culture for an additional 6 hr. DAPI-staining profiles of the cells were then compared with fluorescence profiles of the same cells due to the presence of the DIN7-GFP protein. Din7-GFP shows a punctate pattern coincident with that of DAPI-stained mtDNA (Figure 4). Clearly, Din7-GFP fluorescence does not localize with strong spots of nuclear DAPI staining. These results suggested that Din7-GFP is located in mitochondria. To verify this conclusion, cell fractionation experiments were performed. Strain Y300 carrying either plasmid pPK3 (*GAL1-DIN7*) or vector plasmid pPM29 was grown for ~6 hr in YPGal medium, and mitochondria were isolated from cell extracts. Proteins from mitochondria and cytoplasm were resolved by polyacrylamide gel electrophoresis, and the presence of Din7p was determined by immunoblotting. Since pPK3 carries the *DIN7* sequence fused at the C terminus to the HSV-tag sequence, Din7p was detected with the use of the HSV antibody. Ferrochelatase, an enzyme that is associated with the inner mitochondrial membrane, served as a mitochondrial marker. Hexokinase was used as a cytoplasmic marker. Appropriate cellular fractionation was confirmed by the lack of ferrochelatase in the cytoplasmic fraction and the lack of hexokinase in the mitochondrial fraction (Figure 5). Three polypeptides that reacted with the HSV antibody were found in the mitochondrial fraction derived from Y300 harboring pPK3 (Figure 5). The slowest migrating 48-kD polypeptide is of the predicted size of the tagged version of Din7p. The presence of the smaller polypeptides most likely reflects a rapid degradation of the full-size Din7p. A very similar pattern of proteins was observed in the mitochondrial fractions that were incubated with or without proteinase K, suggesting that Din7p is located inside mitochondria. Din7p was not detected in the cytoplasmic fraction.

Din7p is endowed with nuclease activity: Since the closest homolog of Din7p, ExoI, is a 5′-3′ double-stranded DNA exonuclease (Szankasi and Smith 1992; Fiorentini *et al.* 1997), we wished to examine whether Din7p is endowed with nuclease activity also. We used strain MF21 *exo1::URA3* harboring the plasmid pPK3. The plasmid pPK3 carries a *GAL1-DIN7* sequence fused at the C terminus to the HSV- and hexahistidine-tag sequences. MF21 harboring either pPK3 or vector pPM29 was grown in YNBGal medium, and extracts derived from the cells were partially purified with the use of metal ion chromatography and were assayed for nuclease activity. Table 2 shows that there was a significant induction of the nuclease activity in a partially purified extract prepared from cells carrying pPK3 compared with the control extract. The induced nuclease was active both with the single-stranded (ss) and double-stranded (ds) substrate. Further characterization of the Din7p nuclease activity is in progress.

DISCUSSION

We reported previously that the Din7 protein belongs to a family of *S. cerevisiae* proteins that are involved in

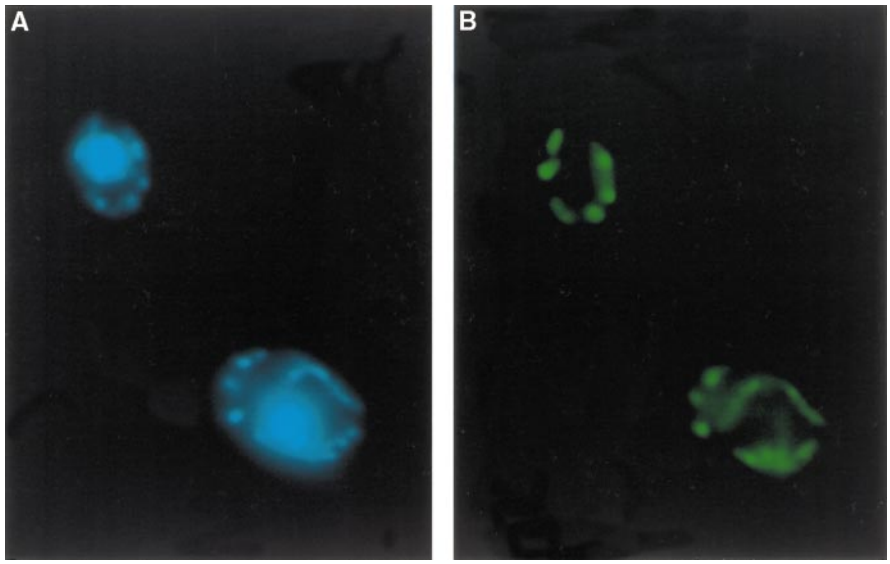

Figure 4.—Localization of the Din7-GFP fusion protein. Strain FY1679 harboring the plasmid pPM39/*din7-GFP* was grown to mid-logarithmic phase in YPG medium, and galactose was added to the culture for 6 hr. DNA was stained by the addition of DAPI (1 μg/ml) for 1 hr, and cells were resuspended in PBS-glycerol. DAPI-staining profiles (A) and fluorescence of the same cells due to the presence of Din7-GFP (B) were visualized in a Nikon microscope equipped with the appropriate filters as described in materials and methods.

DNA repair and replication (Mieczkowski *et al.* 1997). Two members of this family, Rad2p and Rad27p, have demonstrated endonuclease and exonuclease activities (Habraken *et al.* 1993, 1994; Harrington and Lieber 1994a). The closest homolog of Din7p is Exo I/Dhs1. The two proteins exhibit 56% identity and 75% similarity over the N-terminal 367 amino acids. Exo I of both *S. cerevisiae* and *S. pombe* is a 5′-3′ double-stranded DNA exonuclease (Szankasi and Smith 1992; Fiorentini *et al.* 1997). It has been shown that Exo I of *S. cerevisiae* functions in the Msh2-dependent mismatch repair pathway (Tishkoff *et al.* 1997) and is also required for recombination (Fiorentini *et al.* 1997). However, overproduction of Din7p fails to complement the mutator phenotype of the *exo1* mutant, indicating that Din7p cannot substitute for Exo I function in the mismatch repair pathway (P. Mieczkowski and P. Kowprowski, unpublished results). This finding is consistent with previous data (Tishkoff *et al.* 1997) showing that Din7p lacks the domain required for interaction of Exo I with Msh2p and suggests the function of Din7p is different from that of Exo I.

Several of our results indicate that Din7p specifically affects metabolism of mtDNA. First, the increased frequency in the *dun1* strains of the mitochondrial petite (ρ^-) mutants is partially suppressed in strains carrying the *din7::URA3* allele. Second, overproduction of Din7p results in a dramatic increase in the frequency of petites formed. Third, overproduction of Din7p enhances the frequency of mitochondrial mutations conferring resistance to erythromycin (E^r) but does not influence the frequencies of chromosomal mutations conferring either resistance to canavanine (Can^r) or adenine proto-

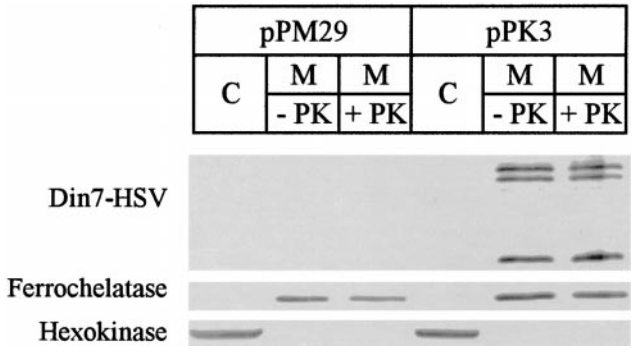

Figure 5.—Subcellular distribution of Din7p. Strain Y300 carrying either the plasmid pPK3(*GAL1-DIN7*) or vector plasmid pPM29 was grown for 6 hr in YPGal medium. Proteins derived from the cytoplasmic (C) and mitochondrial (M) fractions were fractionated on a 10% SDS polyacrylamide gel and tested by Western blotting with the HSV antibody to detect Din7-HSV, and with antiferrochelatase antibody or antihexokinase antibody to detect the mitochondrial or cytoplasmic marker proteins, respectively. The mitochondrial fractions were incubated with (+PK) or without (−PK) proteinase K. Cytoplasmic fractions have 100 μg of protein per lane. Mitochondrial fractions have 5 μg of protein per lane.

TABLE 2

DNA nuclease activity in *S. cerevisiae*

Strain	Substrate	TCA-soluble nucleotides (pmol)
MF21/pPM29	ssDNA	1
MF21/pPK3(*DIN7*)	ssDNA	31
MF21/pPM29	dsDNA	7
MF21/pPK3(*DIN7*)	dsDNA	24

Strain MF21 *exo1::URA3* harboring pPK3, which carries *DIN7* sequence fused to the hexahistidine-tag sequence and the expression of which is under control of the *GAL1* promoter, and MF21 carrying vector pPM29 were grown in YNBG medium. Then, galactose was added for an additional 6 hr. Extracts were prepared and were partially purified with the use of metal ion chromatography as described in materials and methods. Reaction mixtures contained 5 μl (4.1 μg protein) of Ni-NTA agarose fractions eluted with 50 mm imidazole. Activity is expressed as picomoles of TCA-soluble nucleotides released.

trophy (Ade$^+$). Finally, the experiments with the hybrid Din7-GFP protein and cell fractionation experiments indicate that the protein is specifically located in mitochondria.

The increased level of Din7p results in enhanced production of petites that are predominantly ρ^- and not ρ^0 petites. What is the mechanism by which Din7p induces generation of ρ^- mutants? In *S. cerevisiae*, ρ^- mutants result from extensive deletions of wild-type mtDNA sequences (Bernardi 1979; Dujon 1981). The fragment retained after deletion is amplified many times by regular repetitions, forming long DNA molecules that can reach the size of those in the wild-type cell. Thus, the formation of ρ^- mutants involves a mechanism of deletion-amplification rather than just a simple deletion. Sequence analysis carried out to characterize the endpoints of deletion and amplification indicates that intramolecular recombination between short repeats of the ρ^+ mtDNA molecules is responsible for the formation of most ρ^- mutants (Bernardi 1982). It should be emphasized here that the closest homolog of Din7p, Exo I, has been shown to be required for recombination between nontandem direct repeats of the *ade2* gene (Fiorentini *et al.* 1997). Interestingly, it was shown previously that the presence of multiple copies of the *NUC2*/*RNC1* gene, encoding an endo-exonuclease, enhances the spontaneous occurrence of petite mutations (Chow and Kunz 1991). Under *in vitro* conditions, the Nuc2 nuclease is essential for the majority of recombination events when both substrates are double stranded (Moore *et al.* 1993). Thus, it is reasonable to assume that nuclease activity of Din7p may affect recombination of mtDNA. The hypothesis that the increased synthesis of Din7p results in the enhanced recombination of ρ^+ mtDNA is being tested in our laboratory.

The expression of the *DIN7* gene is induced by exposure to DNA-damaging agents such as methyl methanesulfonate (MMS) or UV light (Mieczkowski *et al.* 1997). Interestingly, a constitutive level of *DIN7* expression was found in strains in which the *DUN1* gene was inactivated. Dun1 protein kinase (Zhou and Elledge 1993) is required for efficient induction of the *RNR1*, *RNR2*, and *RNR3* genes, encoding three different subunits of ribonucleotide reductase, one of the key enzymes required for DNA replication and repair since it provides the dNTP pools necessary for DNA synthesis (Elledge and Davis 1987, 1989; Elledge *et al.* 1992). It was reported previously that the *dun1* mutants exhibit increased sensitivity to UV light and MMS (Zhou and Elledge 1993). We show in the present study that the *dun1* strains, compared with the isogenic wild-type strains, exhibit an increased frequency of petite (ρ^-) mutants. The enhanced production of petites is suppressed in *dun1* by multiple copies of the *RNR1* gene, which encodes the large subunit of ribonucleotide reductase, suggesting that this novel phenotype of *dun1* is due to the decreased concentration of dNTP in the mutant cells. Suboptimal concentrations of dNTP in the *dun1* cells may directly affect the efficiency and accuracy of mtDNA replication by the mtDNA polymerase. In consequence, ρ^- mtDNA molecules might be generated more frequently. The observation that a high copy number of the *RNR1* gene decreases the accumulation of ρ^- mutants, arising in diploids harboring a single copy of the *MIP1* gene encoding mtDNA polymerase (Lecrenier and Foury 1995), is consistent with this possibility.

Our observation that in *dun1* petite mutants arise with a high frequency is interesting in light of the recent finding that the frequency of petite formation is correlated positively with the copy number of the *SML1* gene, the product of which inhibits dNTP synthesis by binding directly to Rnr1p (Zhao *et al.* 1998). The frequency of petite formation in *sml1*Δ strains is significantly lower than that of wild-type strains, while expression of an extra copy of *SML1* causes higher frequency of petites. These results indicate that accurate replication of mtDNA is strictly dependent on levels of cellular dNTP pools. Interestingly, it has been shown that *sml1* mutations not only rescue the lethality of *mec1* null strains lacking this essential checkpoint protein (reviewed by Weinert 1998), but can also suppress the increased frequency of petite formation exhibited in *mec1-3* mutants (Zhao *et al.* 1998). According to the model proposed by Zhao *et al.* (1998), Mec1 functions normally to remove the inhibitory effect of Sml1 on Rnr1 during S-phase. The authors suggest that the decreased dNTP pools in *mec1* mutant cells are responsible for defective replication of both chromosomal and mitochondrial DNA.

On the other hand, however, decreased dNTP pools in the *dun1* cells may be responsible for triggering the induction of *DIN7*. This possibility is supported by our finding that the presence of multiple copies of *RNR1* in the *dun1* mutants significantly reduces the basal level of *DIN7* expression (M. Fikus, unpublished results). The enhanced synthesis of the Din7p and, in consequence, its elevated level in mitochondria of the *dun1* mutants may result in a more frequent generation of defective ρ^- mtDNA molecules. Generation of this fraction of ρ^- mtDNA molecules, which are produced in *dun1* by this indirect mechanism, would be prevented by the *din7* mutations.

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